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Fabara, Andrea N; Fraaije, Marco W

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Production of indigo through the use of a dual-function substrate and a bifunctional fusion enzyme

Andrea N. Fabara, Marco W. Fraaije *

Molecular Enzymology Group, University of Groningen, Nijenborgh 4, 9747AG, Groningen, The Netherlands

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ABSTRACT

The current chemical process for industrial indigo production puts a heavy burden on the environment. An attractive option would be to develop an alternative biotechnological process which does not rely on a petrochemical. This study describes a new biotransformation approach in which L-tryptophan is used as starting material. Its conversion to indigo can be achieved through recombinant overexpression of a bifunctional fusion enzyme, flavin-containing monooxygenase (FMO) fused to tryptophanase (TRP). First, TRP converts L-tryptophan into pyruvate, ammonia and indole. The formed indole serves as substrate for FMO, resulting in indigo formation, while pyruvate fuels the cells for regenerating the required NADPH. To optimize this bioconversion, different fusion constructs were tested. Fusing TRP to FMO at either the N-terminus (TRP-FMO) or the C-terminus (FMO-TRP) resulted in similar high expression levels of bifunctional fusion enzymes. Using whole cells and L-tryptophan as a precursor, high production levels of indigo could be obtained, significantly higher when compared with cells containing only overexpressed FMO. The TRP-FMO containing cells gave the highest yield of indigo resulting in full conversion of 2.0 g L-tryptophan into 1.7 g indigo per liter of culture. The process developed in this study provides an alternative biotransformation approach for the production of indigo starting from biobased starting material.

1. Introduction

The beginning of the 20th century saw the development of a petrochemical-based chemical process to synthesize indigo which rapidly replaced the traditional plant-based indigo production [1]. Despite the cost-effectiveness of the process, the hazardous and environmentally unfriendly aspects of the chemical process provide a strong motivation to develop an environmentally benign indigo production process. Biotechnological production of indigo could develop into an attractive alternative approach. The first demonstration of the use of an recombinant microorganism for the fermentative production of indigo was already reported in 1983 [2]. A recombinant *Escherichia coli* strain carrying the *Pseudomonas putida* genes encoding naphthalene dioxygenase (NDO) was used. This process depended on the native *E. coli* tryptophanase converting L-tryptophan to indole, which was then converted to indoxyl by NDO, followed by spontaneous oxidation leading to indigo formation. Later, an improved engineered NDO-expressing strain, with an inactivated trpB gene encoding the β-subunit of L-tryptophan synthase, was shown to produce indigo directly from glucose [3]. Nevertheless, indigo production was found to be limited by the first step of aromatic acids biosynthesis, the formation of 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP). DAHP synthase was found to be inactivated by indigo [4]. To overcome this limitation, a more thorough genetic engineering effort was made to boost the biosynthesis of L-tryptophan, and thus production of indigo [4]. Even though this example showed a great potential, further optimization is necessary to achieve a cost-efficient microbial indigo production. One limiting factor seems to be the rather complex multi-component NDO and formation of unwanted indigoid products.

In the past decades, a large number of alternative natural and engineered enzymes have been identified capable of indigo synthesis through oxidation of indole [5–10]. Many indigo-forming enzymes use a flavin cofactor for the oxygenation reaction [1]. Several class B flavoprotein monooxygenases from bacterial origin, so-called flavin-containing monooxygenases (FMOs), appear attractive biocatalysts as they are single component monooxygenases that merely require NADPH for activity [3,10]. Recently, it has been shown that, after optimization of conditions, indole conversion by FMO-expressing *E. coli*

* Corresponding author.
E-mail address: m.w.fraaije@rug.nl (M.W. Fraaije).

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cells can yield 0.92 g/L of indigo [11]. However, indole is a rather toxic, hazardous and petrol-based chemical. Therefore, we sought to investigate an alternative approach in which L-tryptophan is used as starting material which would allow the development of an indigo production process based on a renewable compound. This aromatic amino acid can be produced through hydrolysis of protein from biomass. Additionally, recent developments in metabolic engineering [12–14] have resulted in various efficient L-tryptophan producing bacterial strains. We explored the use of L-tryptophan as starting material for the design of an effective biotransformation process for indigo production with a bacterial FMO as key indigo-forming enzyme in combination with overexpression of tryptophanase fused to FMO. (Fig. 1)

It is known that *E. coli* is capable of importing L-tryptophan from the medium [11]. Upon import, the native PLP-containing TRP will convert it into indole. In the absence of exogenous added L-tryptophan, *E. coli* has a very limited capacity in producing L-tryptophan and indole. Yet, it was recently shown that by adding L-tryptophan to the medium, relatively high concentrations of indole (5 mM) can be attained [15]. This prompted us to use the combination of TRP and FMO to produce indigo using the non-toxic biobased amino acid L-tryptophan as the starting material. To boost the performance of this couple of enzymes, we decided to express both enzymes fused as a bifunctional fusion enzyme. The possibilities and advantages of enzyme fusions have been explored for various enzymes, including fusions of redox enzymes [16–18]. For example, we have previously fused phosphite dehydrogenase (PTDH) with FMOs, Baeyer-Villiger monooxygenases and a P450 monooxygenase. This resulted in excellent expression levels of soluble and active self-sufficient monooxygenases [19,20]. In fact, Nature often brings enzymes in close proximity through subcellular reaction compartments, membrane-associated complexes on cellular surfaces, scaffold-organized proteins or protein clusters, and modular fusion enzymes [17]. There is compelling evidence showing that organized multienzyme complexes are relatively effective due to advantages such as superior stability, channeling of intermediates and/or simultaneous regulation [16,18].

For our study, we used the FMO from *Methylophaga* sp. strain SK and the TRP from *Escherichia coli*, strain K12. The dimeric soluble NADPH-dependent FAD-containing FMO was previously shown to be efficient in producing indigo from indole [3]. The PLP-dependent TRP catalyzes the hydrolytic β-elimination of L-tryptophan to indole, ammonia and pyruvate [21]. Both possible organizations of the fusion enzymes were constructed, either as TRP-FMO or FMO-TRP. The fused enzymes were expressed, purified and characterized. Using whole cells, expressing the fusion enzymes, conversions of L-tryptophan to indigo were performed and analyzed. Quantification of whole cell based indigo production revealed that TRP-FMO was the most effective construct, producing around 1.7 g of indigo per liter of culture using shake flasks. This work illustrates how fusion enzymes can be employed in whole cells for efficient bioconversion.

2. Materials and methods

2.1. Chemicals, reagents, bacterial strains, and culture conditions

Indigo and indole were purchased from Sigma Aldrich. $NADP^+$ and NADPH were purchased from Oriental yeast Co (Tokyo, Japan). Other chemicals, medium components were purchased from either Sigma-Aldrich, Merck or Thermo Fisher. The protein markers (PageRuler™ Plus Prestained Protein Ladder, 10–250 kDa) were from Thermo Fisher. T4 ligase and the restriction enzyme BsaI were ordered from New England Biolabs. The PfuUltra Hotstart PCR master mix was purchased from Agilent Technologies. *E. coli* NEB 10-beta (New England Biolabs) chemically competent cells were used as a host for cloning the recombinant plasmids, and for protein expression. Precultures were grown in glass tubes with lysogeny broth (LB). For the subsequent main culture, LB supplemented with L-tryptophan was used in baffled flasks.

2.2. Golden gate cloning for FMO-TRP and TRP-FMO

The tryptophanase encoding gene was amplified from genomic DNA isolated from *E. coli* NEB10β using the following primers: forward, GAGGTCTCGCCATATGGAAAACTTTAAAC; reverse GTGGTCTCGCAA GTTAAACTTCTTTAAG). Using Golden Gate cloning, the gene was inserted into pBAD-His vector. The final construct was verified by sequencing. For expressing FMO of *Methylophaga* sp. strain SK1 (AF494423) we used an *E. coli* codon optimized gene synthesized by GeneScript. The FMO and TRP genes with different flanking regions which contain restriction sites were amplified by PCR. The fused constructs FMO-TRP and TRP-FMO were

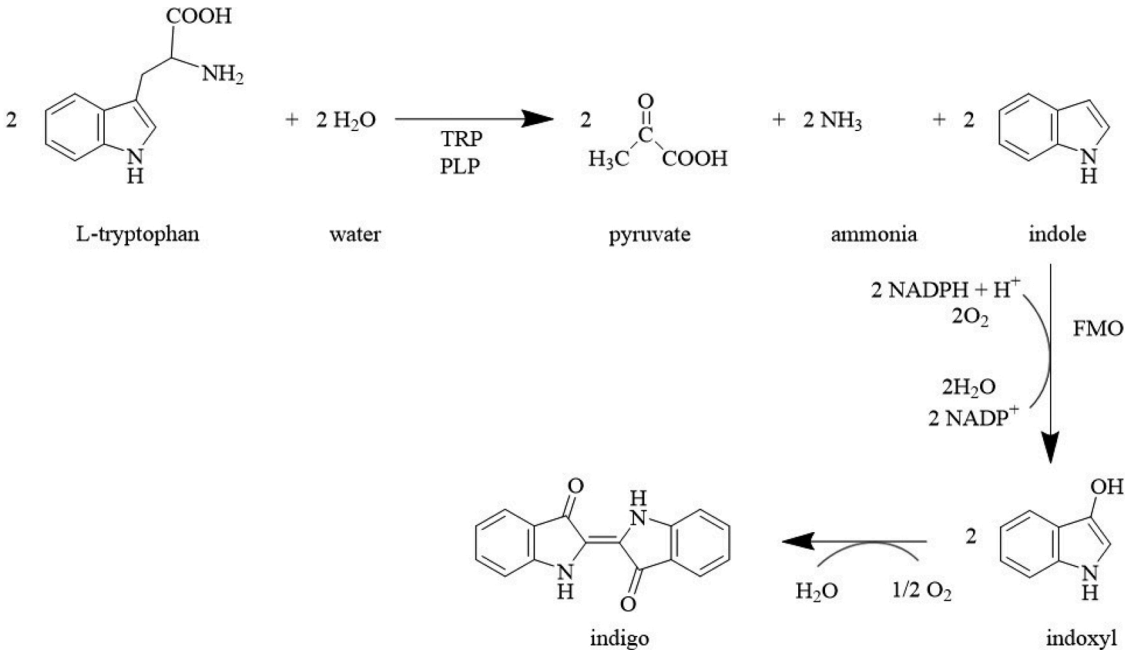

Fig. 1. Using FMO and TRP as biocatalysts, L-tryptophan is converted into indigo via indole and indoxyl as reaction intermediates.

cloned with the Golden Gate cloning technique. Primers used were designed to contain BsaI sites and linker regions (Table 1). The primers were designed such that the PCR products from FMO gene and the TRP gene could be inserted together into a pBAD vector. The pBAD vector contains two BsaI restriction sites, with an upstream region coding for an N-terminal 6xHis tag, an AraC promoter, and an ampicillin resistance gene. To transform host cells with the fusion constructs, the Golden Gate reaction mixture (50 μL) was added to chemically competent *E. coli* cells, and a heat shock (42 °C) was applied for 30 s. After overnight growth on an LB agar plate with ampicillin, colonies were picked and grown in LB, then the plasmids were isolated and sent for sequencing (GATC, Germany) to confirm the correct ligation of the genes.

2.3. Site-directed mutagenesis

The construct for expression of the TRP-FMO* mutant was prepared using the QuickChange kit (Stratagene, La Jolla, CA) (Table 2) [22]. The pBAD-His vector TRP-FMO was used as template DNA. The reactions were carried out in 25 μL of the supplied reaction mixture containing 10 μM of each primer, and ~100 ng/μL of plasmid DNA, DMSO 5%, $MgCl_2$ 50 mM, Pfu Ultra 12.5 μL and MQ water. The PCR cycle involved denaturation at 95 °C for 5 min, then 26 cycles of denaturation at 95 °C for 20 s, annealing 60 °C for 30 s, extension at 72 °C for 1 min/kb of plasmid length, and a final extension at 72 °C for 10 min. PCR products were purified using the PCR Clean-up kit and the template was digested with DpnI at 37 °C for 1 h to remove parental methylated DNA. To transform host cells with the fusion construct, the PCR reaction mixture (10 μL) was added to chemically competent *E. coli* cells, and a heat shock (42 °C) was applied for 30 s. After overnight growth on an LB agar plate with ampicillin, colonies were picked and grown in LB, then the plasmids were isolated and sent for sequencing (GATC, Germany) to confirm the correct ligation of the genes.

2.4. Protein expression and conversions with whole cells

E. coli cells NEB10β transformed with the pBAD FMO-TRP or pBAD TRP-FMO plasmid were grown overnight at 37 °C in 5 mL LB containing 50 μg/mL ampicillin. The precultures were used to inoculate 50 mL of LB supplemented with 2.0 g L-tryptophan/L, containing 50 μg/mL ampicillin and 0.2 % L-arabinose, in 200 mL baffled flasks. The 50 mL cultures were incubated at the indicated temperatures and incubation times. Fusion enzymes were produced and purified as previously described [23].

2.5. SDS-PAGE and UV/Vis spectra

During the purification, samples were taken for SDS-PAGE analysis. SDS loading dye was added to the samples and incubated at 95 °C for 5 min, then centrifuged at 13,000 *g* for 1 min. The samples were loaded onto a precast SDS-PAGE gel (GenScript, USA), and the gels were run in a Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad) at 120 V. The gel was stained with Coomassie InstantBlue (Expedeon, US). An UV/Vis absorption spectrum from 200 to 700 nm (V-330 Spectrophotometer, JASCO) was taken of each purified protein in a quartz cuvette (Hellma Analytics, Germany). The protein concentration for TRP-FMO ($\varepsilon_{433} = 16.9\ mM^{-1}\ cm^{-1}$) was calculated by using the absorbance values at 433 nm.

Table 2
Primers used to obtain site-directed mutants with the mutagenic region in capitals.

Site directed mutants	Primers used to amplify the TRP gene
Double M15 L/S23A	F cgtcaggcCTGgctcaactgcgtgcgtttcaaGCGgctcaggaaaaag
	R tttcctgagcCGCttgaaacgcacgcagttgagccaGgcctgacggac
Single C78I FW	F cgaaagaaATTctggaattcgcagattac
	R gaattccagAATttctttcgggccgtta

2.6. Specific activity determination of PTDH-FMO/ FMO-TRP/ TRP-FMO

For activity measurements and determination of kinetic measurements, FMO activity was measured by following the NADPH depletion at 340 nm ($\varepsilon_{340,NADPH} = 6.22\ mM^{-1}\ cm^{-1}$). After the enzyme (0.5 μM) had been mixed with 4.0 mM indole in buffer (50 mM KPi buffer pH 7.5), NADPH (100 μM, final concentration) was added to start the reaction, briefly mixed in a cuvette, and then the reaction was followed (V-730 Spectrophotometer, JASCO). For TRP activity, kinetic measurements were done by following the NADPH depletion at 340 nm by a coupled enzyme assay using alanine dehydrogenase. After the enzyme (0.5 μM) had been mixed with 10 mM L-tryptophan in buffer (50 mM KPi buffer pH 7.5), 100 μM NADH, 1 mM PLP, and 8 U/mL of alanine dehydrogenase was added and briefly mixed in a cuvette, and then the reaction was followed (V-330 Spectrophotometer, JASCO). The slopes of the initial 40 s were used to calculate the activity rates ($\varepsilon_{340,NADH} = 6.22\ mM^{-1}\ cm^{-1}$). All measurements were made in duplicate or triplicate.

2.7. Indigo quantification

Indigo quantification was based on a method previously described [24]. First, a calibration curve was prepared using cultures with an empty pBAD plasmid and with added amounts of indigo. After the cultures were harvested, indigo was extracted using ethyl acetate up to 6 mg/L. Experiments were performed in triplicates. The 50 mL cultures containing indigo and cells were centrifuged at 4 °C 18,500 × g for 20 min. Then, to liberate all the indigo from the cells, the pellet was resuspended in 10 mL of 50 mM KPi buffer pH 7.5, disrupted by sonication, and the cell-free extract was obtained by centrifugation at 4 °C 18,500 × g for 20 min. From this, the appropriate dilutions were made to dilute with 10 mL of water and extracted with 10 mL ethyl acetate. Indigo content was determined spectrophotometrically at 600 nm.

3. Results and discussion

3.1. Enzyme fusions

We have previously shown that FMO fused to His-tagged phosphite dehydrogenase (PTDH) could be overexpressed to high levels and that the fusion enzyme PTDH-FMO is capable to oxidize indole into indigo

Table 1
Primers used to obtain the fusion enzymes.

Constructs		Primers used
FMO-TRP	FMO	F CGGTCTCGCCATATGGCAACCCGTATCGCTATTC
		R CAATGGTCTCTGCAGAGCCCGATGCTTCTTTCGCCACCGGGATTTC
	TRP	F CAATGGTCTCTGCAGAGCCCGATGCTTCTTTCGCCACCGGGATTTC
		R TGGTCTCTCAAGTTAAACTTCTTTAAGTTTTGCGGTGAAG
TRP-FMO	TRP	F CGGTCTCGCCATATGGAAAACTTTAAACATCTC
		R CAATGGTCTCTGCAGAGCCCGAAACTTCTTTAAGTTTTGCGGTG
	FMO	F TAGGTCTCGCTGCAGCTGGTATGGCAACCCGTATCGCTATTC
		R TGGTCTCTCAAGTTATGCTTCTTTCGCCAC

[25,26]. In this study, PTDH-FMO was produced again and used as a reference fusion enzyme to compare expression levels and the activity of the newly developed fusion enzymes. It is worth noting that the PTDH cannot be exploited in bioconversions because phosphite cannot enter *E. coli* cells. The two new pBAD-based expression constructs for producing TRP-FMO and FMO-TRP were successfully constructed using the Golden Gate cloning method. The two fusion enzymes were equipped with an N-terminal His-tag to facilitate enzyme purification. A short flexible peptide linker (SGSAAG) was used to fuse the two enzymes, which was also successfully used for other fusion enzymes [27]. In our previous study, expression of PTDH-FMO resulted in about 60 mg of purified fusion enzyme per liter of culture [28]. By analogy, we anticipated that overexpression of TRP, an endogenous protein, and its fusion proteins in *E. coli* should be feasible. Expression experiments showed that the two new fusion proteins reached a similar high expression level of soluble protein as compared with PTDH-FMO (Fig. 2).

SDS-PAGE analysis revealed a molecular weight of ~108 kDa for both newly created fusion enzymes, which is in agreement with the theoretical value 108.3 kDa. Both fusion enzymes could be purified by affinity chromatography, resulting in yellow protein fractions. An extinction coefficient of 167 mM^{-1} cm^{-1} at 280 nm for the total protein amount was used to determine the final yield of purified fusion enzyme; that ranged from 20 to 60 mg based on 1 L of culture broth. We investigated whether fusing these oligomeric enzymes (TRP being tetrameric and FMO being dimeric) affected their oligomerization state. For this, the newly designed purified fusion enzymes were analyzed using dynamic light scattering. This revealed that the fusion proteins mainly form oligomers of around 780 kDa (see Fig. 1 Supplementary data). This would correspond with an octamer, possibly composed of two tetrameric TRP modules, each decorated with four FMO dimers.

In order to confirm that the activity of the individual fusion domains was preserved in the fusion enzymes, we first used two different chromogenic tests. To probe FMO activity, the enzymes were incubated with indole and NADPH. A blue color appeared, which demonstrated the conversion of indole to indigo. The second test involved the use of Kovacs reagent which reacts with indole to form a pink-purple adduct [26]. Incubating the fusion enzymes with L-tryptophan and subsequent addition of Kovacs reagent resulted in formation of a clear pink color that confirmed that both fused enzymes were bifunctional. Fusing enzymes may influence the individual specific activities of the two domains. Therefore, FMO and TRP activities were measured and compared to the activities of the purified PTDH-FMO and SUMO-TRP. Furthermore, to investigate the amount of FAD bound to FMO, the purified fusion domains were analyzed by UV–vis spectroscopy. The results revealed that for FMO-TRP, 80 % of the protein did not bind FAD while the other fusion enzymes where mainly purified as holo proteins (Table 3). This also explains the relatively low FMO activity of the purified FMO-TRP. The TRP activities of the two respective fusion enzymes were similar. The measured specific activities were in the same range as reported for the individual enzymes [29,30]. As the fused enzymes mainly retained their catalytic properties, we continued to study their use in the whole-cell conversion of indigo. One should also keep in mind that the enzyme activities may be underestimated as the conditions in the cell may boost enzyme activities. For example, the relatively high intracellular concentrations of the FAD and PLP cofactors will promote holo formation of both enzyme domains. Overall, these results indicated that FMO activity could be the limiting factor for the cascade reaction since the FMO activity of the bifunctional enzymes was relatively low when compared to TRP activity.

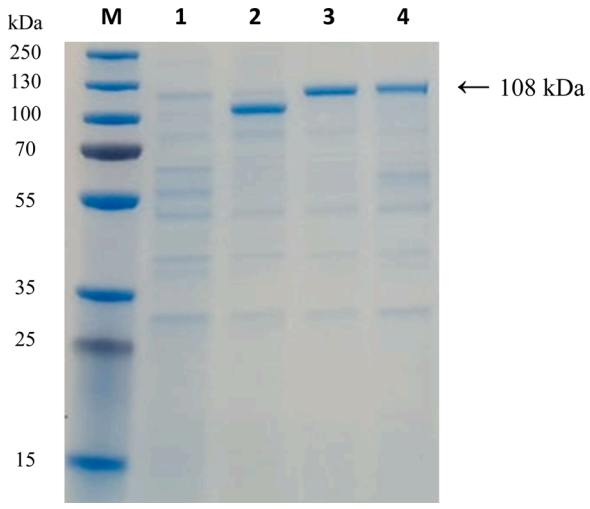

Fig. 2. SDS-PAGE analysis of cleared cell extracts for evaluating the expression of soluble FMO fusion enzymes. Lanes: M, markers; 1, control (empty pBAD); 2, PTDH-FMO; 3, FMO-TRP; 4, TRP-FMO.

3.2. Optimization of medium composition and temperature over time

To identify optimal conditions for indigo production by cultures of TRP-FMO-expressing cells were grown at 24 °C for 40 h and at 30 °C for 24 h using different media (TB, LB or M9 with supplements) with 3.0 g/L L-tryptophan. Indigo production performance was inspected visually. The optimal condition was found to be the use of LB while growing at 24 °C for 40 h. Since the limiting step of the cascade reaction is likely the indole hydroxylation by FMO, we tested the effect of adding riboflavin 50 μg/mL to medium in order to increase intracellular FAD production. Yet, this did not affect the production of indigo. The next step to optimize indigo production was to find the ideal concentration of L-tryptophan. L-tryptophan concentrations were tested from 0.5 to 5.0 g/L and the formed indigo was determined. This revealed that a concentration of 2.0 g/L resulted in the highest indigo concentration (Table 4).

To assess the performance of the different fusion enzymes for indigo production, PTDH-FMO, FMO-TRP and TRP-FMO were independently expressed in *E. coli* cells. Indigo formation in the different cultures supplemented with 2.0 g/L of L-tryptophan was monitored over time (Fig. 3). Cells expressing TRP-FMO produced the most indigo reaching 1.7 g/L after 3.5 days. When considering the molecular masses of indigo and tryptophan, this equals full conversion of the supplied L-tryptophan. Cells expressing FMO-TRP formed significantly less indigo (1.28 g/L) while PTDH-FMO produced even less (0.67 g/L). The lower yield with FMO-TRP is in line with the relative performance of this fusion enzyme upon purification (vide supra). From these results we can conclude that the TRP-FMO fusion enzyme was the best construct since it produced the highest levels of indigo.

A previous study showed that the bacterial FMO could be improved for its performance on indole by introducing structure-inspired mutations. Mutations were identified that increased the thermostability and that improved the k_{cat} for indole [22]. Therefore, we decided to introduce the respective mutations (M15 L/S23A/C78I) in the TRP-FMO fusion. This fusion variant was coined TRP-FMO* and tested for its performance in whole cell conversions of L-tryptophan. This revealed that the TRP-FMO* expressing cells reach a similar amount of indigo when compared with the fusion containing the wild type FMO (1.7 g/L),

Table 3
Specific activities of purified PTDH-FMO, FMO-TRP and TRP-FMO and estimated amount of bound FAD cofactor. For TRP activity, 10 mM L-tryptophan was used, and for FMO activity, 4.0 mM indole was used.

Enzyme	TRP specific activity (U/mg)	FMO specific activity (U/mg)	bound FAD (%)
PTDH-FMO	–	0.6	96
FMO-TRP	1.7	0.2	20
TRP-FMO	1.8	0.4	84

Table 4
Effect of L-tryptophan concentration on indigo production using TRP-FMO expressing cells grown in LB at 24 °C for 40 h.

L-tryptophan (g/L)	Bio-indigo production (g/L)
0.5	0.43
1.5	0.91
2.0	1.30
5.0	0.81

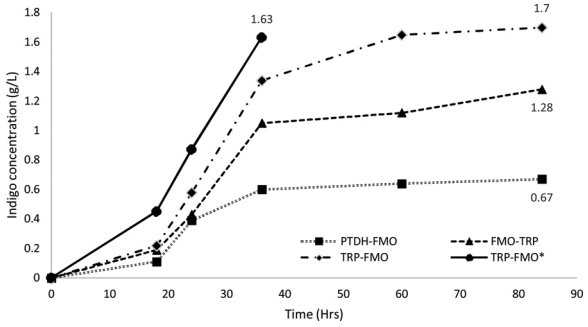

Fig. 3. Analysis of indigo quantification from the different fused enzymes over time.

but in a much shorter time (40 h vs. 84 h, see Fig. 3). Clearly, the engineered FMO domain has a beneficial effect on the performance which confirms that the FMO activity is the limiting factor in converting L-tryptophan into indigo using the recombinant *E. coli* cells.

4. Conclusion

We demonstrated that L-tryptophan could serve as a non-hazardous starting material for indigo production by using TRP/FMO fusion enzymes. The fusion enzymes FMO-TRP and TRP-FMO were efficiently expressed as active bifunctional enzymes. *E. coli* cells expressing these fusion enzymes outperformed cells that only contained FMO activity. By optimizing reaction conditions, full conversion of 2.0 g/L of L-tryptophan could be obtained with cells containing TRP-FMO. The highest efficiency was obtained when using an engineered FMO variant as fusion partner. Except for L-tryptophan, no co-substrate was added to the medium which indicates that the pyruvate that is liberated upon conversion of tryptophan is used for intracellular metabolism that fuels the NADPH-dependent FMO. This makes this biotransformation approach appealing as starting point for developing a biotechnological process for indigo production. A next step in developing such a process is the combined use of the herein developed L-tryptophan-converting *E. coli* with an engineered microorganism that produces L-tryptophan. Alternatively, the developed indigo-producing *E. coli* could be equipped with a potent L-tryptophan synthesis system. By this, effective production of indigo may be achieved which merely requires renewable biomass resources.

Author agreement

Both authors have seen and approved the final version of the manuscript being submitted. We warrant that the article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

CRediT authorship contribution statement

Andrea N. Fabara: Methodology, Validation, Investigation, Project administration, Writing - original draft, Writing - review & editing. **Marco W. Fraaije:** Conceptualization, Supervision, Resources, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.enzmictec.2020.109692.

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